Changes of Electron Spin Resonance Membrane Fluidity in Hexadecane-Induced Hyperproliferative Epidermis

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To study some of the biochemical and physical states of membranes associated with hyperproliferation, the effect of topical hexadecane on membrane fluidity in guinea pig epidermis was investigated by electron spin resonance using a 5-doxylstearic acid spin labeling agent. Guinea pig epidermal cells were separated into three regions of keratinocytes by Percoll density gradient centrifugation. Membrane fluidity and Na^+, K^+-ATPase activity were higher in hyperproliferating epidermal cells than in control. The free cholesterol content and the molar ratio of free cholesterol to phospholipid were found to decrease significantly. Also, elevated levels of palmitic acid, stearic acid and ω-3 unsaturated fatty acid derived from phospholipid were observed. Normal differentiation of epidermis was found to be accompanied by a decrease in membrane fluidity, whereas a relatively high membrane fluidity was maintained in the hexadecane-induced hyperproliferation. *J Invest Dermatol 93:682–686, 1989*

The epidermis is one of the tissues characterized by extensive proliferation and differentiation. The process of cell differentiation in the epidermis is very striking during cellular keratinization. Recently, the physiologic importance of the plasma membrane in regulating intracellular functions has been recognized [1,2]. In our previous papers [3,4], the change of membrane fluidity of epidermal cells during cellular differentiation was investigated by means of electron spin resonance (ESR) using a spin labeling probe. A decrease in the plasma membrane fluidity was found as cells approached the granular cell layer. In the present report, the biochemical and physical states of membranes were further studied in the hyperproliferating and hyperkeratotic epidermis induced by topical application of hexadecane. This study provides basic information for understanding the role of the plasma membrane in the process of keratinization of epidermal cells.

MATERIALS AND METHODS

Animals The skin of guinea pigs (500–600 g) was used. To obtain hyperproliferating tissue, topical application of 25% n-hexadecane petroleum-ether solution was carried out on the first, third, and fifth days of the experiment, using the described method [5]. The seventh day after original application, when the maximum epidermal thickening was observed [5], the guinea pigs were killed and epidermal samples were obtained. Eight animals were used as nontreated control and five animals were treated with n-hexadecane to obtain hyperproliferating epidermis.

Preparation of Epidermal Cells Guinea pigs were anesthetized by a subcutaneous injection of pentobarbital sodium (50 mg/kg body weight), and killed by decapitation. The hair was clipped from the back, and the remaining hair was removed completely with a depilatory cream. After washing the cream from the skin with warm water, skin was excised from the back with scissors. Thin slices (normal skin: 0.3 mm each in thickness; hexadecane-induced hyperproliferating skin: 0.6 mm) were prepared from the excised skin by keratotome. The slices were cut into small pieces, and incubated in Ca^{2+} $-Mg^{2+}$ free phosphate-buffered saline solution [PBS(−), pH 7.4] containing 20 mM EDTA for 60 min at 37°C. The epidermal sheet was peeled from the dermis by forceps and stirred in 0.25% trypsin (1:250, Difco)-PBS(−) solution (pH 7.4) for 30 min at 37°C. The solution was filtered through nylon mesh, and the filtrate was stirred in 0.01% DNase (#D-5025, Sigma Chemical Co.)-PBS(−) solution (pH 7.4) for 10 min at 37°C. The solution was filtered then through a nylon filter (40 μm pore, Thal Ltd. Ca. Swiss), to collect the isolated cells. The cells were washed three times with PBS(−) solution, and the final pellet was obtained by 5-min centrifugation at 700 × g.

Separation of Epidermal Cells by Percoll Gradient Cells obtained from hyperproliferating epidermis and control (nontreated) epidermis were separated into three epidermal cell layers by our technique [3,4], using a slight modification of Slaga's Percoll density gradient centrifugation [6]. The three regions of epidermal cells that formed on the Percoll gradient were divided, using density marker beads (specific density, sp 1.017, 1.049, 1.076, and 1.120; Pharmacia). In our previous paper [7], it was confirmed by light microscope examination that the majority of cells in the low density region (1.017–1.049) were spherical granular cells, those in the middle density region (1.049–1.076) were flattened squamous cells, and those in the high density region (1.076–1.120) were small basal cells. Therefore, the epidermal cells were divided as follows:

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Abbreviations:
C/P ratio: molar ratio of cholesterol to phospholipid
5-DSA: 5-doxyl stearic acid
ESR: electron spin resonance
PBS (−): Ca^{2+}–Mg^{2+} free Dulbecco's phsophate buffered saline solution
sp: specific density

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sp. 1.017–1.049: upper cell layer; sp. 1.049–1.076: middle cell layer; and sp. 1.076–1.120: lower cell layer. The cells from each of the above regions were washed three times with PBS (−) solution, and the respective pellets were used as a sample for ESR and other studies.

Spin Labeling 5-doxyl stearic acid (5-DSA) (Aldrich Chemical Co.) was used as a stearic acid spin labeling probe. The 5-DSA spin label was stored in a freezer at −80°C, as a stock solution containing 0.1 mg/ml ethanol. Fifteen micrograms of 5-DSA in ethanol was placed in a brown-colored conical test tube and dried to a thin film under a flow of nitrogen gas. Three and one-half milliliters of cell suspension (8×10^6 cells) in PBS(−) was prepared for ESR study (the number of cells was adjusted by counting with a homocytometer). The cell suspension was put into the test tube and incubated for 10 min at 37°C with gentle shaking to incorporate the 5-DSA into the cell membrane as described by Murphree et al [8]. Our previous study [9] confirmed that incubation at 37°C for 10 min was sufficient to incorporate the spin label into the cell membrane. The cells were washed with PBS(−) solution to remove the free spin labels, and were centrifuged at 600 × g for 5 min. The pellet then was transferred to a disposable glass capillary (50 μl) and after sealing one end of the capillary with clay, the capillary was placed into the ESR tube. The ESR spectrum was recorded up to 10 min after incorporation of 5-DSA, on a JEOL X-band Spectrometer, model JES-FE3X (JEOL Ltd., Tokyo, Japan) (microwave power, 4 mW; field: 3280 ± 50 gauss; modulation: 100 KHz, 2.0 G; response: 1 s). The cavity temperature was regulated at 37°C with a variable temperature regulator, model JES-ESVT-3A2 (JEOL Ltd.). The ESR spectrum is dependent on the angle between the applied magnetic field and the molecular axes of the nitroxide free radical. Figure 1 shows the ESR spectrum of 5-DSA embedded in the epidermal cells. Anisotropic motion of the spin probe was observed, as 5-DSA was incorporated into a lipid bilayer. Thus, maximal and minimal hyperfine splittings were found. The values of the outer ($2T_{11}$) and inner ($2T_1$) hyperfine splitting were measured in gauss on the ESR spectrum, with an ESR data computer system, model ES-PRIT-33 (JEOL Ltd.).

The order parameter (S) was calculated according to the formula of Gaffney [10]. The order parameter (S) reflects the dynamic state of the phospholipid bilayer [11], the so-called membrane fluidity. The S value shifts between 1.0 and 0.0. A value for order parameter (S) approaching 0 represents high membrane fluidity in the region surrounding the spin probe.

Na^+, K^+-ATPase Na^+, K^+-ATPase activity was assayed using our slight modification [4] of the method of Schimmel [12]. The enzyme activity was determined by the difference between total ATPase (without ouabain) and the activity remaining after the addition of ouabain (1 mM). The activity was expressed as nanomoles inorganic phosphate per milligram of protein per minute liberated into the acid-soluble supernatant.

Cholesterol and Phospholipid Total lipid was extracted from epidermal cells (1×10^7) by the method of Folch et al [13], and was dried with nitrogen gas for analysis of cholesterol and phospholipid contents. Free cholesterol content was determined by Allain's method [14] in the presence of cholesterol oxidase. Phospholipid content was determined by Bartlett's method [15]. To analyze the fatty acid composition of the phospholipid, phospholipid was separated from the total lipid by thin-layer chromatography on precoated silica-gel GF (Merck) with chloroform/ethanol/water/triethylamine [16].

The fatty acid methyl esters were obtained by methanolic hydrocholoric treatment, and were analyzed by gas chromatography on a 10% diethylene glycol succinate polyester column (3.5 mm × 2.5 m) at 210°C with nitrogen gas flow [17]. Heptadecanoic acid (17:1) was used for inner standardization.

Statistical Analysis All data were presented as mean value ± standard deviation. Significant difference were tested by the unpaired Student's *t*-test. The criterion of significance was a *P* value of less than 0.05.

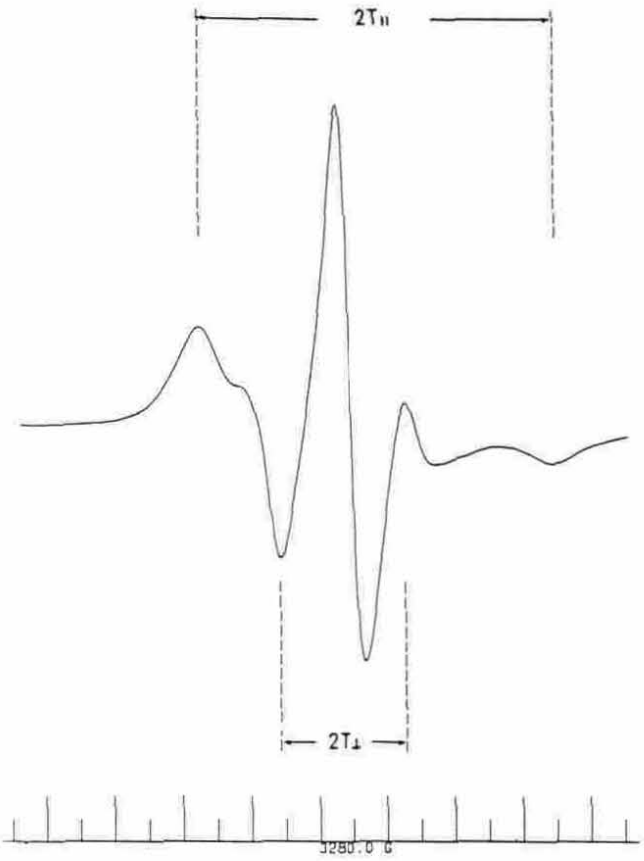

Figure 1. ESR spectrum of 5-doxylstearic acid spin embedded in guinea pig epidermal cells. Temperature: 37°C; microwave power: 4 mW; field: 3280 ± 50 gauss; modulation: 100 KHz, 2.0 gauss; response: 1 s. The order parameter S was calculated, according to the following formula [10]. $S = \frac{T_{\parallel} - T_{\perp} - C}{T_{\parallel} + T_{\perp} + 2C} \times 1.723$, where $C = 1.4 - 0.053\ (T_{\perp} - T_{\parallel})$.

RESULTS

The order parameter S value for the epidermal cells as a whole was 0.631 ± 0.003 (n = 8) for the controls, and 0.612 ± 0.004 (n = 5) for hexadecane-induced hyperproliferating epidermis. Therefore, the membrane of hyperproliferating epidermal cells was found to be less rigid than that of the controls. Figure 2 shows the order parameter S values obtained from cells in the three regions of the epidermis. In the control, the values increase progressively as the cells approach the upper cell layer; namely the normal keratinization of epidermis is accompanied by a decrease in membrane fluidity. In the hyperproliferating epidermis, the values remain at levels lower than those of the control, and the value in the upper cell layer remains at the same level as in the middle cell layer.

As the physical state of membrane fluidity is associated with membrane-bound enzyme activity, Table I shows the activity of Na^+-K^+ ATPase in each of the three layers of epidermis. In the control, the activity decreases gradually from the lower cell layer to the upper cell layer. Activity in the hyperproliferating cells was higher than that in the control, however, no significant difference was detected among the three cell layers. The activity distribution was almost uniform across the layers.

Figure 3 shows the analysis of the lipid content extracted from the cells of the three layers; phospholipid (P) content, free cholesterol (C) content, and the C/P molar ratio. A decrease of free cholesterol content and of the C/P molar ratio was observed in each layer of the

hyperproliferating epidermis, as compared with the control, but no significant difference in phospholipid content was observed.

According to the GC analysis of the fatty acid composition of the phospholipid, increases of percentage composition of palmitic acid (16:0) and of stearic acid (18:0), and a decrease of saturated long-chain fatty acids (20:0, 22:0, and 24:0) was observed as shown in Table II. Regarding unsaturated fatty acid, a decrease of monounsaturated fatty acids and an increase of polyunsaturated fatty acids was detected. In particular, the ω-3 unsaturated fatty acid population increased significantly.

DISCUSSION

ESR spectra obtained from cell membrane are known to decay spontaneously. The spin label nitroxide group is reduced by reducing agents in the membranes and cytoplasm [18]. To avoid the spin label reduction, ESR is measured up to 10 min after the incorporation of the spin label into the membrane. The spontaneous decay of the 5-DSA ESR intensity was not observed significantly until 60 min after the incorporation. However, the cells lost $48.2 \pm 1.7\%$ of their signal intensity after 3 h at 37°C. After incubation with the membrane impermeable oxidizing agent $K_3Fe(CN)_6$ [19], the intensity was found to recover to $90.1 \pm 9.1\%$ of the original intensity. These observations indicate that 5-DSA remains predominantly in the plasma membrane.

Topical application of hexadecane induced typical hyperproliferation and hyperkeratosis in the epidermis [5,20]. The maximum hyperproliferation was observed 1 wk after the original topical application under our procedure [5]. The hexadecane-induced hyperproliferating epidermal cells retained a relatively high membrane fluidity. Kimelberg et al [21] indicated that free cholesterol in the lipid bilayer reduced the membrane fluidity of phospholipid. In our present study, the free cholesterol content was found to decline in the hyperproliferating epidermis. The percentage composition of polyunsaturated fatty acids was found to increase. In a separate experiment [22], it was observed that the exposure of B-16 melanoma cells to ultraviolet irradiation resulted in a biphasic alteration of membrane fluidity. The membrane fluidity was found to increase 6 h postexposure. The analysis of lipid content 6 h postexposure showed decreases of free cholesterol content and of the C/P molar ratio. Increases in the double-bond index and of polyunsaturated fatty acid content were also observed. This pattern was similar to that obtained in the present experiment. Brasitus et al [23] determined the lipid composition and fluidity of brushborder membrane prepared from rat proximal and distal colonocytes. Fluidity measured by the fluorescense polarization technique was found to decrease in the distal as compared with the proximal plasma membranes. The decrease in fluidity of the distal as compared with the proximal membrane resulted from an increase in cholesterol content and in the cholesterol ratio. As for the fatty acid composition of phospholipid, the ω-3 unsaturated fatty acids increased in the hyperproliferative epidermis. From these results, the change of membrane fluidity can be attributed to differences in the phospholipid composition.

Table I. Na^+, K^+-ATPase Activity in n-Hexadecane-Induced Hyperproliferative Epidermis of Guinea Pig

	Control (n:8)	Hyperproliferative epidermis (n:5)
Upper cell layer	1.07 ± 0.18^a	5.08 ± 1.08
Middle cell layer	1.26 ± 0.15^a	5.78 ± 0.95
Lower cell layer	1.94 ± 0.45	4.31 ± 0.55

Values are means ± SD.
[a] Significant difference for $p < 0.05$ versus values of lower cell layer.

Na^+, K^+-ATPase is a typical marker enzyme embedded in the plasma membrane. As reported by Kimelberg et al [21], Na^+, K^+-ATPase is strongly influenced by changes in membrane fluidity. Keeffe et al [24] found that changes of liver plasma membrane fluidity parallel those in Na^+, K^+-ATPase activity, with measurement by the fluorescence polarization technique. The membrane fluidity correlated with Na^+, K^+-ATPase activity, but was not correlated with Mg-ATPase or 5′-nucleotidase activity. Observations by Van Zoelen et al [25] on exponentially growing Neuro-2A cells have shown that plasma membrane bound Na^+, K^+-ATPase activity

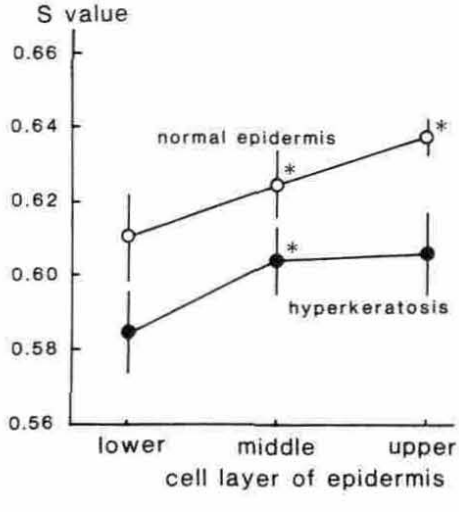

Figure 2. Order parameter S value of 5-doxylstearic acid incorporated into the plasma membrane of guinea pig epidermal cells. The ordinate indicates the order parameter S value. The S value shifts between 1.0 and 0.0. Low values represent high fluidity in the region surrounding the spin probe. * Significant differences for $p < 0.05$ versus values of lower and middle cell layer, respectively.

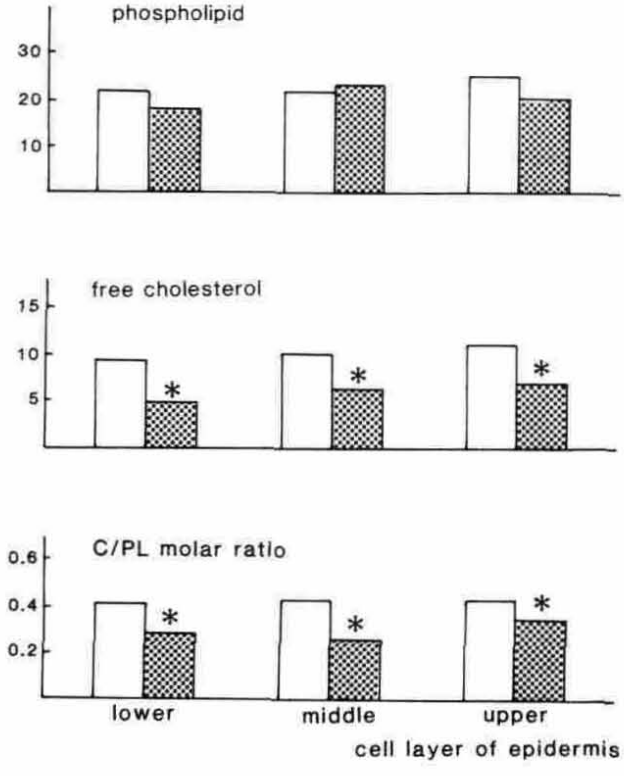

Figure 3. Phospholipid composition derived from control and n-hexadecane-induced hyperproliferative epidermis. Phospholipid and free cholesterol contents were expressed as nano moles per mg protein. * Significant differences for $p < 0.05$ versus normal epidermal cells. *Open bars* = control; *hatched bars* = hyperproliferative epidermis.

Table II. Fatty Acid Composition of Phospholipid in the n-Hexadecane-Induced Hyperproliferative Epidermis

	Control			Hyperproliferative epidermis		
	Lower	Middle	Upper	Lower	Middle	Upper
C16:0	8.5	8.5	8.5	10.3*	11.0*	10.7*
C16:1	4.2	4.5	4.6	3.5*	4.0	3.8
C18:0	8.1	8.2	8.2	10.4	9.8*	10.2*
C18:1	51.8	50.9	51.0	51.2	48.9	50.0
C18:2	10.7	10.6	10.7	11.0	11.9	10.7
C18:3	0.6	0.4	0.5	0.7	0.7*	0.7*
C20:0	0.6	0.8	0.7	0.4	0.5	0.5
C20:1	1.6	1.8	1.7	1.5	1.5*	1.6
C20:4	1.3	1.0	1.1	1.0*	1.2	0.9
C22:0	1.2	1.7	0.5	0.6	0.7*	0.7*
C22:5	0.2	0.3	0.3	0.1	0.1*	0.1*
C22:6	0.1	0.2	0.1	0.4*	0.5*	0.4*
C24:0	1.6	2.4	2.4	0.9	1.1	1.4*
$\omega-6$	12.1	11.7	11.9	12.1	13.3*	11.7
$\omega-3$	0.7	0.6	0.6	1.1*	1.2*	1.2*
Saturated	21.0	22.3	22.2	23.7	24.1	24.8*
Unsaturated	74.3	73.3	73.8	73.8	72.8	72.3
Monoenoic	59.6	59.0	59.2	58.3	56.4*	57.4
Polyenoic	16.3	16.1	16.3	16.9	17.9*	16.5
Double-bond index	0.91	0.89	0.90	0.92	0.92	0.90

*p (<0.05:versus normal epidermal cells. $n = 5$.

shows considerable variation during the cell cycle. The modulation of membrane fluidity was found to regulate the Na^+, K^+-ATPase activity. In fact, Na^+, K^+-ATPase activity in guinea pig epidermis was found to decrease gradually as the cells approached the upper layer, and this decrease was accompanied by a decrease of membrane fluidity [4]. In normal epidermis, enzyme activity is inversely proportional to membrane fluidity. The differentiation of epidermal cells in the normal keratinization process is associated with a decrease in membrane fluidity and with a decline of Na^+, K^+ ATPase activity. In the hexadecane-induced hyperproliferating epidermis, both membrane fluidity and Na^+, K^+-ATPase activity are higher than those in the control. The membrane fluidity does not decrease progressively as the cells approach the upper layer, but rather remains at the same level in both the middle and upper cell layers. In addition, the distribution pattern of Na^+, K^+-ATPase activity is different from that of control. The distribution was almost uniform across the upper and middle layers of epidermis.

In summary, the hexadecane-induced hyperproliferation caused increases of membrane fluidity and of Na^+, K^+-ATPase activity, and therefore was not a simple enhancement of the pattern in the normal epidermis. The fatty acid composition of phospholipid was significantly different than that of the control.

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